the power of absorbing the ultra-violet rays of the spectrum, though they are inferior in this respect to benzene and its derivatives.

2. Terpenes, with composition $C_{15}H_{24}$, have a greatly increased absorptive power.

3. Neither the terpenes themselves nor their oxides nor hydrates, exhibit absorption bands under any circumstances when pure, but always transmit continuous spectra.

4. Isomeric terpenes transmit spectra which generally differ from one another in length, or show variations on dilution.

5. The process of diluting with alcohol enables the presence of bodies of the aromatic series to be detected in essential oils; and even in some cases the amount of these substances present may be approximately determined.

Researches into the Colouring-matters of Human Urine, with an Account of the Separation of Urobilin." By Chas. A. MacMunn, B.A., M.D. Communicated by A. Gamgee, M.D., F.R.S., Brackenbury Professor of Practical Physiology and Histology in Owens College, Manchester. Received March 6, 1880. Read March 18, 1880.

I do not propose to discuss in this paper all the pigments which have been said to occur in urine, as their consideration would extend over a considerable space; and I shall, therefore, limit my observations to those which I have myself studied, and which are discoverable by means of spectroscopic observation. Notwithstanding the efforts of physiological chemists, at home and abroad, no one has been hitherto able to isolate the pigment known as urobilin. After many unsuccessful attempts, I have at length succeeded in isolating a pigment, which, on account of its spectroscopic and chemical reactions, appears to be urobilin in a pure state.

Although it has not been obtained in sufficient quantity to allow of a formula being assigned to it, I believe that I shall soon be able to obtain enough for this purpose. A preliminary examination has shown that it contains carbon, hydrogen, oxygen, and nitrogen.

Preliminary Remarks.—If nitric acid be added to a solution of bile before the slit of the spectroscope, the solution at once undergoes a change of colour, becoming green, blue, violet, red, and, lastly, yellow or brownish-yellow, and the spectrum is characterised by having two bands: a broad shadowy band, composed of two in orange and yellow, and a black band at Fraunhofer's line F. In a short time the shading in orange and yellow begins to fade, and at the time the oxidation process is completed, and the colour of the solution

The Royal Society is collaborating with JSTOR to digitize, preserve, and extend access to *Proceedings of the Royal Society of London.* www.jstor.org

has become yellow, nothing but the band at F is left. Jaffé ("Zeitsch. f. Chem.," v, 666) succeeded in isolating the pigment which gives the feeble bands on each side of D, and also that which gives the band at F. The pigment which gave this last band, when isolated, was brown-red in colour, soluble in alcohol, ether, and chloroform; the solutions

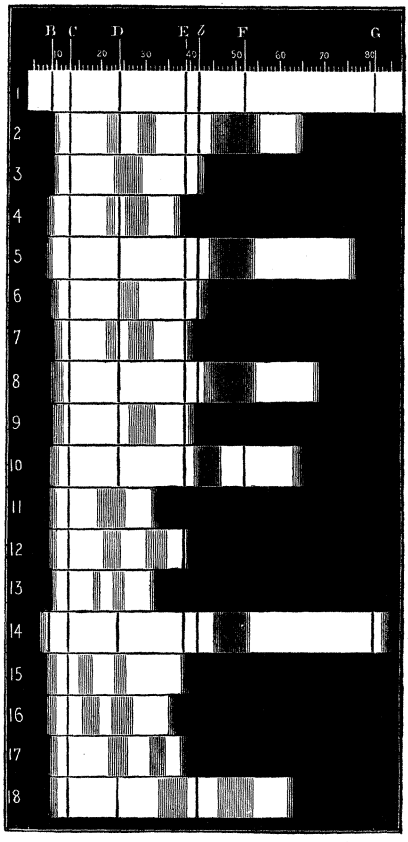

Figure reduced from Chart I. (See p. 36.)

being a fine red colour, giving, when acidulated, a dark band at F. By treating a solution of dog's bile with hydrochloric acid, the same observer obtained a red fluid, which became yellow on the addition of alkalies, and which, before alkalies were added, was marked by giving the same dark band as before, but after the addition of alkalies, especially caustic soda, this band was replaced by another one nearer the red. Since Jaffé described these appearances, Maly* has asserted

* "Ann. Ch. Pharm.," clxi, p. 368; clxiii, p. 77.

that he can produce urobilin, or, as he called it, hydrobilirubin from bilirubin, the red colouring matter of human bile;* and, although his researches have been called in question by some physiological chemists who profess to have repeated his experiments with a negative result, yet the conclusions drawn by him are practically correct, and have led, in my hands, to the discovery of urobilin in the bile of various animals, in human urine, and to its complete isolation from the latter fluid.

Most specimens of high-coloured urine, provided the high colour is not due to blood or unchanged bilirubin or biliverdin, show, when examined with the spectroscope, a dark band at F, which disappears completely when the urine is treated with ammonia, and which is moved towards the red end of the spectrum by treatment with solution of caustic soda. The band can be made to reappear after the ammonia treatment by the addition of nitric, hydrochloric, or acetic acid.

Since almost all specimens of urine which I examined showed a band at F, I had concluded that this band was due to urobilin, but I could not account for the fact that ammonia did not always cause its disappearance. I now find that there are *two pigments in urine which give a band at F.* This discovery was made while I was engaged in studying urobilin, for, on treating normal human urine, which was of a pale straw colour, in the same manner as that adopted for the separation of urobilin, I found a pigment of a brownish colour, which, when dissolved in ether, gave two faint bands, one of which was placed over F, the other between *b* and D (see 18 of figure). The band at F was not made to disappear by ammonia, nor did acids intensify it. As a similar band is found in blood serum, in yelks of eggs, in butter, cheese, &c., which is due to Thudichum's "lutein," I see no objection in accepting that author's name, urolutein, for this pigment. My object in mentioning this is to call attention to the fact that urobilin is not the *only* pigment which gives a band at F; but urobilin appears to be the only pigment which behaves on treatment with acids and alkalies in the manner I have described. The presence of this pigment urolutein in urine containing urobilin as well, has also led to the statement which appears in some text-books of physiological chemistry, that caustic soda, when added to urine containing urobilin, causes *two* bands to appear. The reason is, that caustic soda moves the urobilin band towards the red, but leaves untouched the band of urolutein; consequently, two bands are seen instead of one. When urobilin alone is present, ammonia causes the complete disappearance of the band at F, and caustic soda moves it towards the red. Accordingly, a preliminary examination will enable us to determine whether

* By reduction with sodium amalgam and subsequent treatment with hydrochloric acid.

urobilin alone is present, and the neglect of this observation has led to disappointment.

Examination of the Bile-Spectra of various Animals.—If urobilin be formed from bile, the question naturally arises, is urobilin present as such in the bile of any animal? If so, its biliary origin is made more certain. To enable me to reply to this question, I proceeded to examine this fluid in various animals, using in each instance fresh bile, and examining the spectra by means of a Sorby-Browning micro-spectroscope, and checking observations—when the amount of fluid at my disposal was sufficient for the purpose—by means of a one-prism chemical spectroscope.

The bile of the following animals was examined:—man, pig, dog, cat, guinea-pig, rabbit, mouse, sheep, hedge-hog, ox, crow, blackbird, chicken, goose, wild duck, duck, frog.

Among these animals, the bile of the following gave a characteristic spectrum:—guinea-pig, rabbit, mouse, sheep, ox, crow.

The darkest green or golden-red bile gave the least characteristic spectrum. I have fully described, in the "Spectroscope in Medicine," the colour and spectrum of each specimen, but I shall here merely mention those facts which throw light upon the origin of urobilin, namely, that by careful dilution or by examination in a sufficiently thin depth, a band at F is always visible, that this band is made darker by acids, and is made to disappear by adding ammonia. This is very striking in the case of the bile of the mouse (Chart II, sp. 12*), which gives a black band, resembling exactly that seen in febrile human urine. If Chart II be inspected, the most noticeable appearance is the presence of this band in so many spectra. And one cannot help also noticing the general resemblance between these bile-spectra and those shown in the figure, which are the spectra of urobilin in various solutions, and treated by various reagents.

I also attempted to extract urobilin from the liver of the pig, by means of various solvents, the pounded liver having been extracted with water, alcohol, ether, chloroform, and acidulated alcohol respectively, but without success.

Separation of Urobilin from Human Urine.—Before describing the method which I adopted for the separation of urobilin, I may make one or two preliminary observations. The reagents used were perfectly pure, and pure ethyl alcohol was used in every instance; this is a matter of great importance, as methylated spirit is not suitable for the separation of such easily decomposed bodies as urobilin.

The readings of the spectra are those of a photographed scale adapted to the micro-spectroscope; and before taking the readings, the precaution was always adopted of narrowing the slit until the sodium

* The Chart II referred to above is reproduced in the "Spectroscope in Medicine;" and accordingly does not accompany this paper.

line stood at the same number on the scale. Of course this would have been unnecessary if the jaws of the slit had been made to open equally.

The readings are also given in wave-lengths, the latter having been calculated by means of an interpolation curve; they are expressed in millionths of a millimetre.

Since preliminary observation had shown that there are two pigments in urine which give a band at F, it was necessary, if possible, to get a specimen of urine which contained urobilin only. Accordingly, the urine of a case of phthisis was chosen, which gave the band at F, in very slight depths; this band could be made to disappear by ammonia, and was moved towards the red by caustic soda. The colour of the urine was orange-red. The urine therefore contained urobilin, and on six different occasions I separated urobilin by the following method from it; on each occasion the result was the same. I may mention that no play of colours was produced by nitric acid (containing nitrous.)

Experiments.—230 cub. centims. of urine was precipitated by neutral lead acetate, and filtered, the filtrate which still showed the urobilin band was precipitated with basic lead acetate and filtered; the filtrate now showed no band. The precipitates were united and extracted with alcohol acidulated with sulphuric acid, and again thrown on a filter. The filtrate was a fine, clear red fluid giving in slight depths a well-marked black band at F. Some of this fluid was put into a separating funnel, a large quantity of water added, and then chloroform; the whole well shaken and allowed to stand, the red chloroform layer separated off and examined, when it was seen to give the same black band. The remainder of the alcoholic fluid was treated in the same manner. I had now a fine red fluid, being a solution of urobilin in chloroform, with, however, a little turbidity at its surface, which disappeared after filtration, which was twice repeated.

An attempt was made to separate some of the colouring matter from the chloroform with acidulated water, but with a negative result.

The chloroform was now distilled off, the residue redissolved in chloroform, which was again distilled off (in both cases over a water-bath). The residue was brown-red in colour, glistening on the surface, perfectly amorphous, and gave, when examined on the stage of the micro-spectroscope, with a strong light condensed upon its surface, a black band at F. It is perfectly soluble in alcohol, chloroform, nitric acid, hydrochloric acid, acetic acid, lactic acid, acidulated water, partially in ether, both ethereal solution and residue when dissolved in alcohol, giving same spectrum; partially in benzol and in water; insoluble in bisulphide of carbon.

The chloroformic solution gives no precipitate with chloride of barium, nor after treatment with ammonia.

Ethereal Solution of Urobilin, got by Sulphuric Acid and Alcohol Method described above.—The colour is dark red.

Spectrum—

Extent, 10 to 65 (or wave-length 678—457).
Band ϵ, 21 to 23 (or wave-length 604—592.)
Band δ, 28 to 32 (or wave-length 568—552).
Band a, 45 to 55·5 (or wave-length 507—479)

See figure p. 27 (2).

By treating an appropriate depth of this fluid in a cell with ammonia, the colour becomes lighter and more yellow, and gives the spectrum shown in figure (3). At a less depth, the band at F is seen to have disappeared. This band shown in the figure (2) reads from 23 to 29 (or wave-length 592—564).

When excess of ammonia was removed by means of a small pipette, and the solution was acidified with acetic acid, the band at F reappeared.

Alcoholic Solution of same Pigment.—This solution was of a red colour, and gave, when examined at a suitable depth, two bands, and at a less depth another black band at F.

The readings of the bands at the first depth were as follows:—

Extent, 9 to 37 (or wave-length 686—534)
Band ϵ, 21 to 23 (or wave-length 604—592)
Band δ, 26 to 29 (or wave-length 578—564)

See figure (4).

Less depth:—

Extent, 9 to 78 (or wave-length 686—435)
Band a, 45 to 55 (or wave-length 507—480)

See figure (5).

When the first depth was treated with ammonia, a band appeared reading 25—29 (or wave-length 582—564), and in a slight depth of fluid so treated the band at F had disappeared.

This solution then gives the same spectrum as the ethereal solution, the slight differences in positions of bands being due to different refractive power of solvent used, and when ammonia was added, the same effect as in the case of the ethereal solution was produced. See figure (6.)

Chloroformic Solution.—Red solution, giving in sufficient depth two bands, and in less depth, one band.

The readings of the spectrum of first depth were as follows:—

Extent, 11 to 40 (or wave-length 671—523)
Band ϵ, 21 to 23·5 (or wave-length 604—590)
Band δ, 27 to 31 (or wave-length 573—556)

See figure (7).

The band of the shallower depth read from 44 to 55 (or wave-length 510—480). See figure (8).

Treated with ammonia, I got the band shown in figure (9), reading

from 28—32 (or wave-length 568—552). So that this solution gives practically the same spectrum as the ethereal and the alcoholic.

Benzolic Solution.—Reddish solution giving following spectrum :—

Extent, 11 to 65 (or wave-length 671—457).
Band ϵ, 21 to 23 (or wave-length 604—592).
Band δ, 28 to 32 (or wave-length 568—552).
Band α, 45 to 55 (or wave-length 507—480).

This also gave a band near D when treated with ammonia, which also caused the disappearance of band α.

At first it seemed possible that the appearance of these bands ϵ and δ might have been due to the action of heat on the pigment during the evaporation, but on evaporating the chloroformic solution under the receiver of the air-pump the same result was arrived at.

Action of Caustic Soda on Solutions.—If the solutions were examined in a depth sufficient to show band at F distinctly and caustic soda were added, in each case this band disappeared and was replaced by another nearer the red. Thus in ethereal solution the band read 42—47 (or wave-length 517—502); and in chloroformic solution the band read 43—48 (or wave-length 513—499); and in alcoholic solution 41·5—47 (or wave-length 519—502.

Aqueous Solution.—The aqueous solution gave the black band at F, disappearing with ammonia and moving towards the red with caustic soda.

Solution of Pigment in Hydrochloric Acid.—It dissolved completely in hydrochloric acid, giving a red solution, which in a sufficient depth showed a band at D, figure (11).

Extent, 9 to 32 (or wave-length 686—552).
Band γ, 20 to 25 (or wave-length 609—582).

In less depth :—

Extent, 9 to 63 (or wave-length 686—462).
Band α, 45 to 53 (or wave-length 507—485).

By the ammonia and caustic soda treatment, the spectra were altered in the same manner as those of the other solutions.

Solution of Pigment in Sulphuric Acid.—A red solution.

Deep layer :—

Extent, 8 to 37 (or wave-length 694—534).
Band, 21 to 25 (or wave-length 604—582).

Shallower depth :—

Extent, 7 to 80 (or wave-length 702—432).
Band, 43 to 52 (or wave-length 513—488).

As all these solutions contained the same pigment, the next step

was to determine the elementary composition of this pigment; accordingly the residue from the chloroformic treatment was carefully analysed, when it was found to contain :—

Carbon.
Hydrogen.
Oxygen.
Nitrogen.
Sulphur.

The occurrence of the last element led to the supposition that it must have been due to the treatment with sulphuric acid. I therefore came to the conclusion that if this supposition were correct there would be an absence of sulphur in the pigment prepared by a method in which hydrochloric acid would be used instead of sulphuric.

Separation of Urobilin by means of Hydrochloric Acid.—This method was exactly similar to the first, with the exception, that instead of using alcohol acidulated with sulphuric acid, I now used alcohol acidulated with hydrochloric acid, and the pigment was separated on four different occasions; the residue obtained resembled exactly that got by the first method, both as to its appearance and as to its solubility in different media.

The spectra of these solutions differed (as I expected they would) in regard to the band near D, but that at F was exactly the same as before, and acted in the same manner when treated by ammonia and sodic hydrate respectively.

I now proceed to the spectra observed in the solutions of urobilin obtained by this method.

Alcoholic Solution.—An orange-red solution, which in sufficient depth gave the following spectrum :—

Extent, 10 to 32 (or wave-length 678—552)
Band δ, 18 to 20 (or wave-length 620—609)
Band (feeble) ϵ, 22 to 25 (or wave-length 598—582) } See figure (13).

Shallower depth :—

Extent, 7 to 83 (or wave-length 702—427)
Band, 47 to 54 (or wave-length 502—483) } See figure (14).

Treated with ammonia the colour of the solution became less red, and the following spectrum was seen :—

Extent, 10 to 40 (or wave-length 678—523)
Band δ, 15 to 19 (or wave-length 640—614)
Band δ, 23 to 26·5 (or wave-length 592—576) } See figure (15).

In shallower depths the band α of original solution had disappeared.

Ethereal solution.—Of an orange colour. It gave the following spectrum when treated with ammonia:—

Extent, 10 to 37 (or wave-length 678—534)
Band δ, 16 to 20 (or wave-length 634—609)
Band γ, 23 to 28 (or wave-length 592—568)
} See figure (16).

In a less depth the band α at F of original solution had disappeared.

Chloroformic solution.—This solution gave a similar spectrum to, and was altered similarly by, ammonia as the other solutions just described.

Action of Caustic Soda on these solutions.—In appropriate depths caustic soda moves the band at F nearer the red. Thus in the case of the alcoholic solution:—

Band *before* treatment with caustic soda, 47 to 53 (or wave-length 502—485).

Band *after* treatment with caustic soda, 40 to 45·5 (or wave-length 523—506).

And the action of caustic soda on the ethereal and chloroformic solutions was similar. When urobilin is thus treated with caustic soda, ammonia is no longer capable of causing the band to disappear.

Action of Acids on Urobilin prepared as above.—It dissolves in *sulphuric acid*, giving a splendid ruby-red solution, having the following spectrum:—

Extent, 10 to 40 (or wave-length 678—523).

As the bands are uncertain, alcohol was added, when a band appeared from

21 to 25 (or wave-length 604—582),

and a very faint shading from

30 to 35 (or wave-length 560—542) see figure (12). (Band at F in shallow depth.)

It dissolves in *acetic acid*, forming an orange-red solution, giving in suitable depths the following spectrum:—

Extent, 10 to 38 (or wave-length 678—530)
Band δ, 22 to 26 (or wave-length 598—578)
Band γ, 32 to 35 (or wave-length 552—542)
} See figure (17).

Shallow depths:—

Extent, 8 to 85 (or wave-length 694—425).
Band α, 45 to 55 (or wave-length 507—480).

Ammonia develops in this solution three feeble bands:—

Extent, 10 to 41 (or wave-length 678—520).
Band ε, 8 to 11 (or wave-length 694—671).
Band η, very feeble, reading could not be taken.
Band δ, 34 to 37 (or wave-length 545—534).

In a slight depth the band at F had disappeared. The pigment was also soluble in nitric acid and in lactic acid; in the former there were no bands, except that at F, visible; in the latter they resembled those of the acetic acid solution.

All the spectra which I have described will suffice to show that the same pigment was evidently present in every solution; there were many more observed and measured, but they were not of sufficient importance to call for their being mentioned here.

It now became necessary to test the urobilin prepared by the hydrochloric acid process for sulphur, which was accordingly done; and not only was sulphur found to be absent, but the presence of chlorine was detected, showing that my inference was correct, and that the sulphur found in the urobilin prepared by the sulphuric acid process was due to the sulphuric acid used in its preparation, and that the chlorine found in the urobilin prepared by the hydrochloric acid process was due to the hydrochloric acid. But in neither case was there free sulphuric acid or free hydrochloric acid, and hence the conclusion follows that the urobilin was in combination with those acids respectively.

I think it will be allowed that the bands visible only at certain depths of the solution belong to the same pigment that gives the band at F, hence they all belong to urobilin. As they are not visible in the aqueous solution, we can understand why they are not visible in urine. And I may also mention that, although water only appears to take up a pigment giving a band at F, yet after evaporation of the water and solution of the residue in alcohol, ether, or chloroform, the same bands again become visible.

Summary.

1. Urobilin has been separated from urine.

2. It has been separated in combination with hydrochloric acid and with sulphuric acid respectively.

3. The spectra of solutions of urobilin obtained by these methods respectively differ in the position of certain feeble bands, but agree in all having a black band at F, which can be made to disappear on adding ammonia in excess, and which is replaced by another band nearer the red end of the spectrum on the addition of sodic hydrate.

4. Urobilin is an amorphous brownish-red pigment, which contains carbon, oxygen, hydrogen, and nitrogen. It is soluble in alcohol, chloroform, acidulated water, acids; partially in ether, benzol, and water, *i.e.*, if the pigment be separated in combination with hydrochloric or sulphuric acid.

5. Urobilin appears capable of existing in different states of oxidation.

6. Urobilin is derived from one of the colouring matters of bile.

7. Urobilin is the colouring matter of the bile of the mouse.

Before concluding this paper I should like to call attention to a peculiarity which the band α of urobilin exhibits: in certain depths it appears very broad, but in less depths it is seen that the portion of the band nearest the violet has disappeared. In other words, the redward part of the band is the most persistent and is the last to disappear on dilution. Thus, taking the alcoholic solution of urobilin prepared by the second process mentioned before, we find that when the extent of the spectrum is 8 to 80 (or wave-length 694—432) this band reads 42—67 (or wave-length 517—453), but when the extent is 7 to 85 (or wave-length 702—425) then the band reads 47 to 53 (or wave-length 502—485.) Urobilin, like hæmaglobin and hæmatin, appears to be a very unstable body, which easily splits up on treatment with reagents into decomposition products, each giving a peculiar spectrum. This accounts for the differences observed in the spectra obtained by the different methods I have described.

EXPLANATION OF FIGURE (p. 27).

1. Solar spectrum.
2. Urobilin, prepared by the alcohol and sulphuric acid method, ethereal solution.
3. The same treated with ammonia.
4. Urobilin, prepared by the same method, dissolved in alcohol.
5. Slight depth of the same.
6. Solution, of which 4 is the spectrum, treated with ammonia.
7. Urobilin, prepared as in 2 and 4, dissolved in chloroform.
8. Shallow depth of the same.
9. Solution, of which 7 is the spectrum, treated with ammonia. Solutions 3, 6, and 9, if examined in a shallow depth, show no band at F.
10. Action of caustic soda on slight depth of solution in 2, 4, and 7.
11. The pigment, prepared by the above method, dissolved in hydrochloric acid.
12. Urobilin, prepared by the hydrochloric acid and alcohol method, dissolved in sulphuric acid; at a shallower depth we get the band at F.
13. Urobilin, prepared by the hydrochloric acid and alcohol method, in alcoholic solution.
14. Shallow depth of same.
15. Solution mapped in 13 treated with ammonia.
16. Ethereal solution of urobilin, prepared as in 13 and treated with ammonia.
17. Urobilin, prepared by the same method, dissolved in acetic acid. At less depth band at F is seen.
18. Spectrum from residue,—got by treatment adopted for separation of urobilin,—from pale straw-coloured urine.

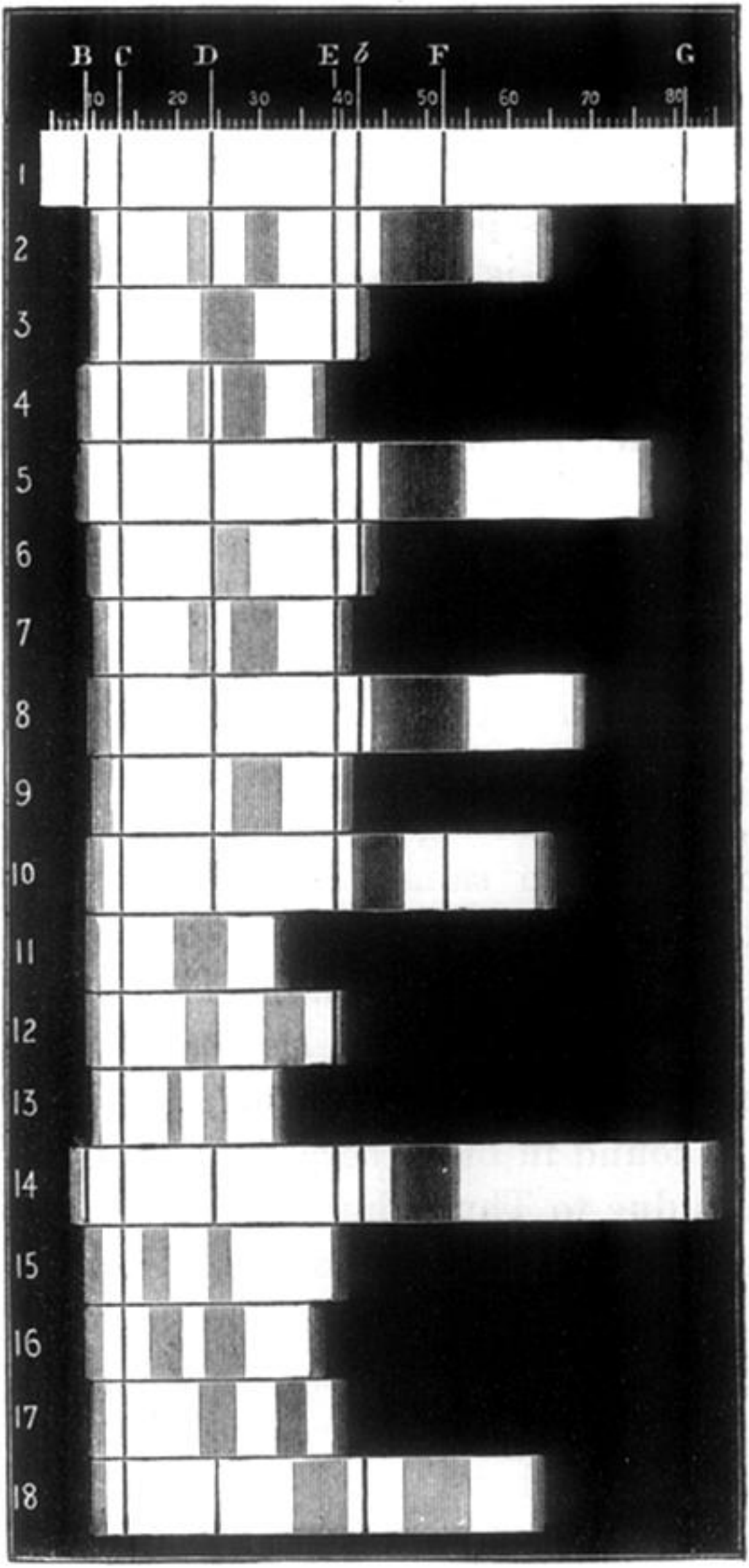

Figure reduced from Chart I. (See p. 36.)